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## Use of immunoelectron microscopy to show Ebola virus during the 1989 United States epizootic

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### Abstract

A filovirus, serologically related to Ebola virus, was detected by "post-embedding" immunoelectron microscopical examination of MA-104 cells. These had been infected by inoculation with serum samples obtained during the 1989 epizootic in cynomolgus monkeys (*Macaca fascicularis*), imported from the Philippines and maintained at Reston, Virginia, USA, a primate holding facility. The immunoelectron microscopy method, when used in conjunction with standard transmission electron microscopy (TEM) of infected cells, provided consistent results and was simple to perform in this epizootic. It is concluded that immunoelectron microscopy is potentially useful in the direct immunological diagnosis of Ebola and related filoviral infections (such as Marburg) in clinical samples obtained from those with acute infection.

Immunoelectron microscopy has been successfully used to identify several viral isolates which pose a threat to public health.<sup>1-3</sup> This paper reports the successful application of a post-embedding immunoelectron microscopy technique to improve the investigation of an epizootic involving a filovirus closely related to Ebola virus<sup>4</sup> in a group of cynomolgus monkeys imported from the Philippines into Reston, Virginia, in November, 1989. Early in the course of the reported investigation,<sup>4</sup> viral particles, morphologically identical with filoviruses, were visualised in thin sections of tissue culture cells inoculated with sera and organ homogenates from infected monkeys. Identification of the isolates was based on immunofluorescence of the inoculated cells plus detection of Ebola viral antigens by antigen capture enzyme-linked immunosorbent assay techniques. Use of immunoelectron microscopy showed that the filovirus particles reacted with Ebola virus reference reagents. The isolation of an Ebola-like virus from non-human primates is without precedent, but subsequently, an independent filovirus isolate was obtained from a second primate holding facility in Philadelphia, Pennsylvania.<sup>5</sup>

### Methods

The history of the monkeys used in this study has been described previously.<sup>4</sup> Serum samples obtained from four sick monkeys at the

Reston facility were diluted 1 in 5 in Eagle's minimal essential medium with Earl's salts (EMEM) and 10% heat inactivated fetal bovine serum. The samples were adsorbed in 0.5 ml aliquots on to confluent monolayers of MA-104 cells (an African green monkey kidney cell line; Whittaker Bioproducts, Walkersville, Maryland, USA) grown in 25 cm<sup>2</sup> tissue culture flasks. The inoculum was adsorbed for one hour at 37°C. About 4.5 ml of EMEM containing 10% heat inactivated fetal bovine serum and 0.1% gentamycin was added to the 0.5 ml aliquot in each flask, the caps were tightened, and the flasks incubated at 37°C for up to 10 days. Cells were examined daily by phase contrast microscopy. When the cytopathic effect reached 3-4+, usually four to five days after inoculation, the cells were harvested for standard transmission electron microscopy and post-embedding immunoelectron microscopy. Uninfected MA-104 cells were harvested and processed in the same way as negative controls.

About 12 cm<sup>2</sup> of MA-104 cells were scraped into 3 ml of culture fluid, divided between two 1.5 cc conical Eppendorf tubes, one for standard transmission electron microscopy and the other for immunoelectron microscopy, and centrifuged for 30 seconds at 12 000 rpm to form a loose pellet of cells. The supernatant was removed and 2% glutaraldehyde in Millonig's phosphate buffer (pH 7.4) was slowly added to the transmission electron microscopy pellets. Paraformaldehyde (2%)-glutaraldehyde (0.1%) in 0.01 M phosphate buffered saline (PBS) (pH 7.2) was added to the immunoelectron microscopy pellets. After fixation for one hour, the pellets for transmission electron microscopy were post-fixed in 1% osmium tetroxide, stained en bloc with 0.5% ethanolic uranyl acetate, dehydrated in ethanol and propylene oxide, embedded in Poly/Bed 812 resin (Polysciences, Inc., Warrington, Pennsylvania, USA), and cured for 18 hours at 60°C. After fixation for 90 minutes the immunoelectron microscopy pellets were sterilised at 4°C by  $2.5 \times 10^6$  rads of gamma irradiation. The immunoelectron microscopy pellets were processed in the same manner as the transmission electron microscopy pellets except they were not post-fixed in osmium tetroxide nor stained with uranyl acetate. In addition to Poly/Bed 812, the immunoelectron microscopy pellets were embedded in LR White resin (Polysciences) and cured at 49°C for 18 hours.

Thin sections for immunoelectron microscopy were collected on 300-mesh nickel electron microscopy grids and processed by an

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Figure 1 High magnification of an Ebola virion (longitudinal section) from MA-104 cell pellet harvested five days after inoculation. Particle is about 950 nm in length. Poly Bed 812 thin section stained with 5% uranyl acetate in 50% methanol and Reynold's lead citrate.



adaptation of the method used by Roth *et al.*<sup>6</sup> For immunogold staining, the grids were completely immersed in 4% normal goat serum (Janssen Life Sciences Products, Piscataway, New Jersey, USA) for 15 minutes at 23°C and rinsed four times with 0.02 M TRIS (hydroxy methyl) amino methane buffer (pH 7.2). The grids were incubated for 16 hours at 4°C in dilutions (1 in 100) of Marburg or Ebola murine monoclonal antibodies (mouse ascitic fluids from hybridoma cells provided by the Centers for Disease Control, Atlanta, Georgia, USA) in TRIS-buffer containing 0.1% bovine serum albumin and 0.05% Tween-20 (pH = 8.1). Ebola murine monoclonal antibodies used included DA1A5B11,

DD4AESA11, MD4B07AE11, and MBA06C08A. The DA1A5B11 monoclonal reacts with the envelope glycoprotein of the Ebola-Sudan strain, while DD4AESA11, MD4B07AE11, and MBA06C08A react with nucleocapsid complexes of both the Sudan and Zaire strains of Ebola virus. A mixture of murine monoclonal antibodies raised to the structural proteins of Marburg virus consisted of CB01BB01, AA05BC03, and FB05BG10. After primary antibody incubation the grids were jet-washed with TRIS-bovine serum albumin-Tween buffer (pH 7.2) for a total of five minutes and transferred to drops of anti-mouse IgG labelled with 10 nm gold spheres (Janssen Life Sciences), diluted 1 in 10 in TRIS-buffer containing 0.1% bovine serum albumin and 0.05% Tween-20 (pH = 8.1), for one hour at 23°C. The grids were rinsed three times with TRIS-bovine serum albumin-Tween buffer, twice with 0.01 M PBS, fixed with 2% glutaraldehyde in PBS, jet-washed three times in distilled water, and stained with uranyl acetate and lead citrate. Grids were examined with a Jeol 100CX electron microscope (Peabody, Minnesota, USA) at 80 kilovolts. Thin sections for standard transmission electron microscopy were collected on 200-mesh copper electron microscopy grids, stained in uranyl acetate and lead citrate, and examined at 80 kilovolts.

## Results

MA-104 cells inoculated with serum samples from the four monkeys (O10B, O53, T20 and T28) were harvested when the cytopathic effect was 3-4+. Monkeys O10B and O53 maintained in room F of the Reston facility were reportedly infected by a filovirus, based on morphology.<sup>4</sup> A filovirus was also observed in thin sections from MA-104 pellets inoculated with serum samples from monkeys T20 and T28, housed in room H of the Reston facility, that had no direct contact with room F monkeys. Transmission electron microscopy of inoculated cell pellets from the room H monkeys showed the presence of filoviral particles that averaged 950 nm in length, 80 nm in cross section, with a nucleocapsid of about 45 nm (figs 1 and 2). Prominent inclusions formed by large aggregates of nucleocapsid were observed in the cytoplasm of infected cells (fig 3).

Immunoelectron microscopy staining of corresponding MA-104 pellets from three of the four monkeys identified the Reston Virginia isolate as a strain of Ebola virus (EBO-R). Figure 4 shows positive immunogold labelling of a nucleocapsid inclusion in an inoculated MA-104 cell, embedded in LR White, incubated with a mixture of murine monoclonal Ebola antibodies. In contrast, fig 5 shows extracellular virions labelled with the same mixture of Ebola specific monoclonal antibodies. Figure 6 shows the reaction of the virions with a single murine monoclonal antibody raised to Ebola-Sudan glycoprotein. The degree of labelling observed was not intense, but in control sections background staining was

Figure 2 Ebola virions, from MA-104 cell pellet, budding from surface of intracytoplasmic vacuole. Cross sections average 80 nm in diameter with nucleocapsids of 45 nm. Method as for figure 1.



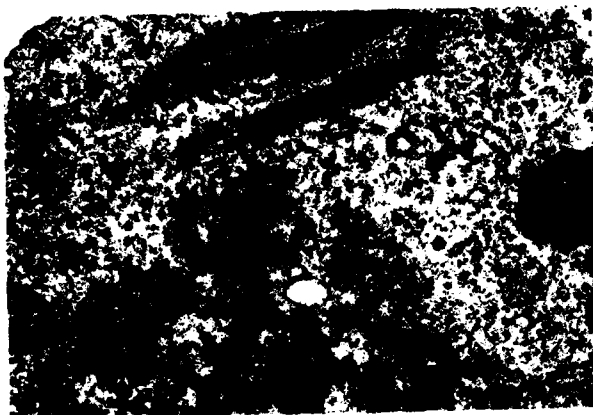


Figure 3 Representative field of inoculated MA-104 cell pellet showing cytoplasmic inclusions formed by nucleocapsids (open arrowheads). Method as for figure 1.



Figure 4 Micrograph showing positive immunogold staining (arrows) of a nucleocapsid inclusion in a MA-104 cell inoculated with a mixture of Ebola monoclonal antibodies (containing monoclonal reactive with both Ebola prototypes). LR White thin section stained with 5% uranyl acetate in 50% methanol and Reynold's lead citrate.



Figure 5 Positive gold sphere staining of the virions (arrowheads) when incubated with a mixture of Ebola monoclonal antibodies (containing monoclonal with reactivity to both Ebola prototypes). Method as for figure 4.

negligible. For example, fig 7 shows an Ebola-infected MA-104 cell with no reaction after incubation with a mixture of Marburg monoclonal antibodies. The Marburg monoclonal antibodies used in fig 7 were reactive with homologous Marburg virus by the immunofluorescence antibody test.<sup>4</sup> Uninfected MA-104 cells did not label when incubated with the Ebola or Marburg antibody mixtures.

Poly/Bed 812 resin provided a higher degree of ultrastructural preservation than LR White resin, and positive immunostaining was demonstrable when it was used. Non-specific background staining, however, was prevalent in cells embedded in Poly/Bed 812, regardless of the concentration of monoclonal antibodies used. Reproducibility of results was difficult and immunostaining varied between regions on the same grid. In contrast, cells embedded in LR White gave uniform results and reduced non-specific background staining even when high concentrations of monoclonal antibodies were used, 1 in 50, for example.

#### Discussion

The post-embedding immunoelectron microscopy technique described corroborated the immunofluorescence antibody test reported previously<sup>4</sup> to identify EBO-R viral antigens in inoculated MA-104 cell cultures. Because prototype filoviruses are established human pathogens,<sup>7,8</sup> the immunoelectron microscopy methodology should assist in the differential diagnosis of suspected viral haemorrhagic fevers in man. A primary advantage of this technique is that it can be performed on tissue culture cells inoculated with serum samples, reducing the risks inherent in handling biopsied tissue specimens. The immunoelectron microscopy technique, however, is being adapted to biopsy specimens and to direct diagnosis of virus in infected sera in our laboratory.

Until the recent EBO-R epizootic<sup>9</sup> Vero cells had been the cells most widely used for the propagation of filoviruses.<sup>10,11</sup> Our findings based on data obtained from MA-104 and Vero cultures inoculated with EBO-R suggest that MA-104 cells may be a more efficient substrate for primary filoviral isolation (unpublished data). Results from this study also suggest that embedding samples in LR White is better than embedding in Poly/Bed 812 for immunoelectron microscopy purposes. This could be because the required polymerisation temperature for Poly/Bed 812 is higher (60°C) than that for LR White (49°C). A change in the antigen binding sites may be a greater problem at the higher temperature.

Immunoelectron microscopy should facilitate epidemiological and experimental investigations, including those for determining the unidentified reservoir of Ebola virus. The association between the EBO-R isolate and the prototype Ebola subtypes has yet to be determined, but data obtained using the post-embedding immunoelectron microscopy technique have shown that there is a definite



Figure 6 Identification of filovirus virions serologically reactive with an Ebola-Sudan monoclonal antibody (DA1ASB11) by the immunogold staining technique. Method as for figure 4.



Figure 7 Control section of Ebola infected MA-104 cells incubated with a mixture of Marburg monoclonal antibodies. Neither the nucleocapsid inclusion (open arrowheads) nor the extracellular virions (arrows), were labelled with heterologous (Marburg-specific) monoclonal antibodies. Method as for figure 4.

reaction with the Ebola-Sudan strain. Further studies are being undertaken to determine the association between EBO-R and the prototype EBO strains. Immunoelectron microscopy has the potential to differentiate among antigenically related filoviruses that share some, but not all, epitopes.

As outbreaks of Ebola-related viruses are a major public health concern and have recently been reported in primate holding facilities,<sup>4,5</sup> techniques that efficiently detect and identify these viruses are urgently needed. Because of the demonstrable use of the post-embedding immunoelectron microscopy method in the EBO-R epizootic, it should be considered for inclusion in the standard panel of rapid diagnostic procedures routinely used for the timely identification of naturally occurring filoviruses.<sup>12</sup> Immunoelectron microscopy should be a useful adjunct to the conventional immunofluorescence antibody test and the newly developed antigen-capture, enzyme-linked immunosorbent assay for Ebola virus.<sup>4</sup>

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